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## Modes of adherence of *Helicobacter pylori* to gastric surface epithelium in gastroduodenal disease: A possible sequence of events leading to internalisation

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We have investigated various modes of adherence of *Helicobacter pylori* to the human gastric epithelium, using transmission electron microscopy, in biopsies from nine patients with peptic ulcer disease and from four patients with chronic active gastritis. *H. pylori* was demonstrated in abundance in all cases within the surface mucous layer. In all ulcer- and in one out of four gastritis patients *H. pylori* was shown in close proximity to the gastric epithelium, with concurrent alterations in the configuration of microvilli and the apical cytoplasmic region of gastric cells. Previously described modes of *H. pylori* adherence were confirmed, such as loose attachment with fibrillar-like strands, firm attachment with pedestal formation, invasion in the intercellular spaces, and invagination with “cup” formation. Moreover, in many cases a fusion between the bacterial outer layer and gastric cell membranes was evident. In four cases (31%; three with active and one with past ulcer disease) viable *H. pylori* was found in the cytoplasm of gastric mucous cells. Our results support the hypothesis that the different modes of adherence of *H. pylori* represent a stepwise, possibly sequential, process which in a significant number of cases leads to internalisation of the organism. The invariable occurrence of adherence and more frequent internalisation of *H. pylori* in ulcer patients may suggest a link with the pathogenesis of peptic ulcer disease.

**Key words:** *Helicobacter pylori*; electron microscopy; peptic ulcer disease; adherence.

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Adherence to target cells is a common property of many bacterial species and a central process in the pathogenesis of various disease conditions (1). It is also important for bacterial entry into epithelial cells (2). Several ultrastructural studies of *Helicobacter pylori* have been conducted during the last few years in the

search for different patterns of adherence to the surface of gastric epithelium which might eventually explain the various mechanisms of virulence. In general terms, cell adhesion or adherence was defined either as a close attachment between the organism and epithelial cells without a visible intervening space (3) or as a “loose” attachment to microvilli.

Earlier ultrastructural studies (4–6) demonstrated many *H. pylori* organisms, often ar-

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TABLE 1. Characteristics of the 13 patients and results of TEM

Case	Age*	Sex	Histopathological diagnosis	TEM findings
1	29	M	Duodenal ulcer	Adhesion type 1-4, internalisation
2	33	M	Duodenal ulcer	Adhesion type 1-4
3	39	M	Deformed duodenal bulb <sup>#</sup>	Adhesion type 1-4, internalisation
4	45	F	Gastritis	No adhesion
5	49	M	Gastritis	No adhesion
6	50	M	Gastritis	Adhesion type 1-3
7	50	F	Gastric end bulb ulcers	Adhesion type 1-4
8	54	F	Gastritis	No adhesion
9	63	M	Antral ulcer	Adhesion type 1-4
10	73	F	Gastric ulcer	Adhesion type 1-4
11	73	F	Gastric ulcer	Adhesion type 1-4, internalisation
12	76	M	Duodenal ulcer	Adhesion type 1-4, internalisation
13	83	M	Duodenal ulcer	Adhesion type 1-4

\*Age at diagnosis. <sup>#</sup> The patient had histological signs of chronic active pancreatitis.

Adhesion types: 1. fibrillar-like strands, "loose" adhesion; 2. invasion of intercellular space; 3. "firm" attachment with pedestal and/or cup formation; 4. fusion of outer bacterial cell layer and gastric membranes.

ranged in groups close to intercellular junctions, which were thought to be attributable to artefacts induced during preparation. Hazell *et al.* (5) speculated that *H. pylori* accumulate in this location to use nutrients available there. However, this was disputed by others (7) who claimed that *H. pylori* was mostly located within the pit mucosa close to the epithelial surface. The organisms were never observed between, underneath or within cells of gastric mucosa, and the relationship between *H. pylori* and the pathological changes was obscure even when the organisms were in close contact with the gastric surface epithelial cell. However, it must be noted that these investigators studied patients with dyspeptic symptoms without evidence of gastric or duodenal ulcer disease. Further studies revealed various modes of cell adhesion (3, 8-12) and according to some reports *H. pylori* was demonstrated in close proximity to parietal cells and even inside the canaliculi of resting (gastric) cells (4, 8, 10, 13, 14).

The possibility of internalisation of *H. pylori* is a matter of debate. The prevailing concept is that *H. pylori* is a non-invasive bacterium (15, 16). In fact, some studies have failed to demonstrate intracellular bacteria, mostly using tumour cell lines, for example gastric adenocarcinoma (17), cervical adenocarcinoma (18) or colon cancer cell monolayers (19). The interpretation of these results is not always straightforward. Hazell *et al.* (5) as well as Thomsen *et al.* (7) found no evidence of invasion of gastric cells by *H. pylori* in biopsies from gastritis patients. At the same time, however, Hazell *et al.* (7) consistently observed penetration of the bacteria in the deep parts of the intercellular junctions. In contrast, many reports have demonstrated occasional (6, 8, 10, 11, 14, 20, 21) or frequent (12) internalisation of *H. pylori* in gastric mucous cells (8, 10, 11, 14, 20), parietal cells (4, 14, 20) or various cell lines (12, 21, 22). The intracellular organisms were mostly viable and sometimes associated

*Fig. 1.* *H. pylori* in the surface mucous layer. A polyphosphate energy structure is evident at one end of the largest organism. TEM  $\times 15,000$ .

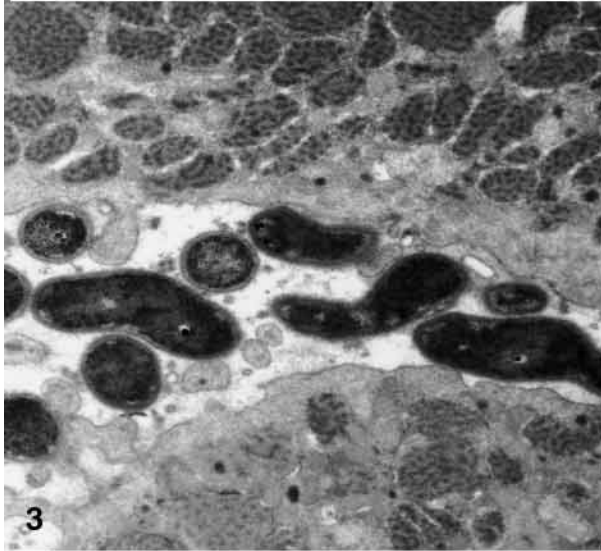
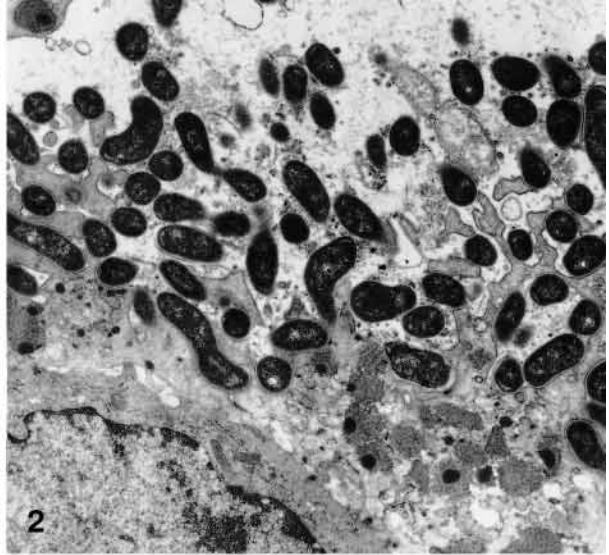
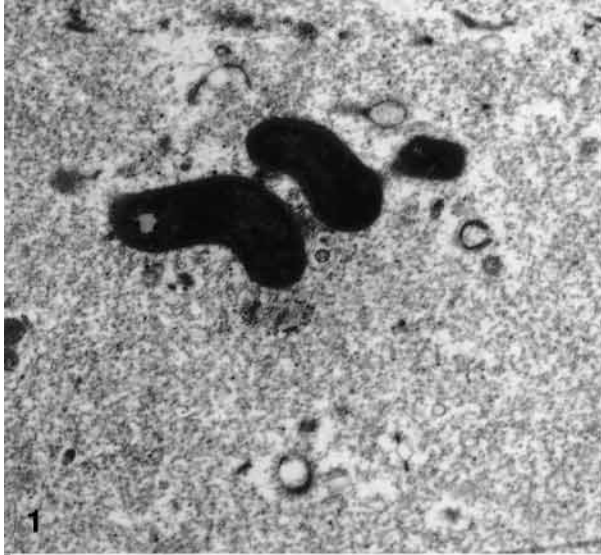
*Fig. 2.* Abundant *H. pylori* in close proximity to the surface of gastric epithelial cells. TEM  $\times 18,000$ .

*Fig. 3.* *H. pylori* within intercellular junctions. Some organisms show adhesion to the gastric cells. TEM  $\times 12,000$ .

*Fig. 4.* Adhesion of *H. pylori* with broad apposition area; microvilli appear deformed and blunted. TEM  $\times 24,000$ .

*Fig. 5.* *H. pylori* invaginated by gastric epithelial cells. Simultaneous loose attachment via multiple fibrilla-like strands. TEM  $\times 39,000$ .

*Fig. 6.* Adhesion of *H. pylori* via central pedestal formation. The organism is in addition adhering to gastric cells with both poles. TEM  $\times 39,000$ .





with mucous droplets or granules (23) or vacuole spaces (22). Even the coccoid forms of the bacterium were found intracellularly (12, 24), strongly indicating that this presence is a true biological phenomenon rather than an artefact.

The present investigation was conducted in order to study the various modes of adherence and the possible relationship to the presence of (type B) gastritis or/and ulcer disease. We also wanted to find out if internalisation of *H. pylori* occurs and if it may play a role in the pathogenicity of the organism.

## MATERIALS AND METHODS

Between 1986 and 1996 13 patients with upper gastrointestinal symptoms were randomly selected at Huddinge and Lund Hospitals. The patients, eight males and five females, ranged in age from 29 to 83 years with an average of 55.2 years (Table 1). At endoscopy, two biopsies were taken from standard localisations, according to the Sydney classification, from the antral and fundic mucosa, for routine histopathological examination. For electron microscopy, 1–2 biopsies from the antral and corpus areas were studied as well as 1–2 biopsies from ulcer areas. Further biopsies were taken from lesions situated in other areas of the gastric, duodenal and esophageal mucosa. Biopsies were fixed in 10% neutral-buffered formalin, processed routinely, embedded in paraffin, and sectioned at 4 µm. Slides were stained with hematoxylin and eosin for routine histopathology and with modified Giemsa to better visualize *H. pylori*. Reporting on gastritis and colonisation by *H. pylori* was based on the Sydney classification.

### *Transmission electron microscopy (TEM)*

For electron microscopy, the biopsies were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.1 M sucrose at pH 7.2, postfixed in 2% osmium tetroxide and s-collidine pH 7.4, dehydrated in graded ethanol, and embedded in Agar 100 resin. 50 nm ultrathin sections were cut on a Reichert-Jung Super Nova ultratome and picked up on formvar-coated 200-mesh copper grids. The grids were stained with 4% uranyl acetate followed by Reynolds lead citrate. The ultrathin sections were examined in a Philips CM 10 at 60 kV.

## RESULTS

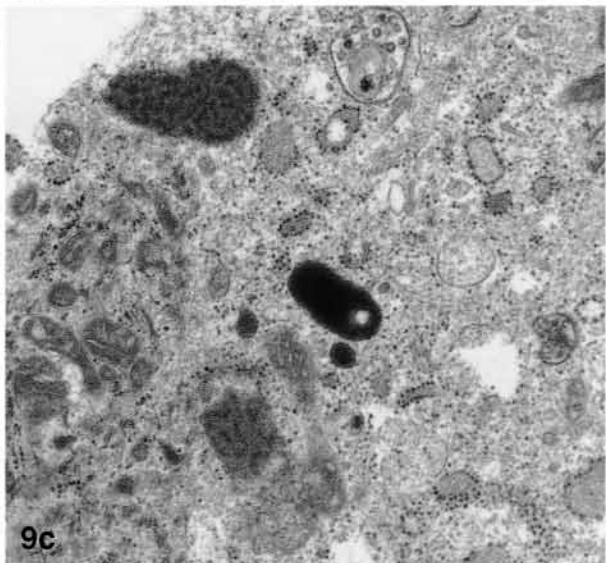
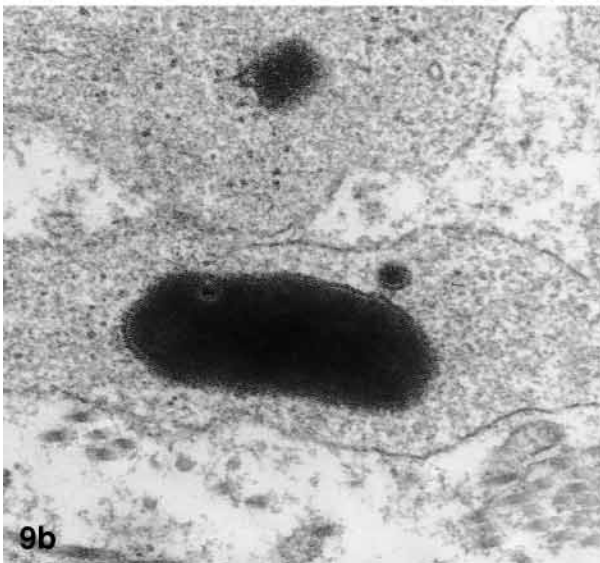
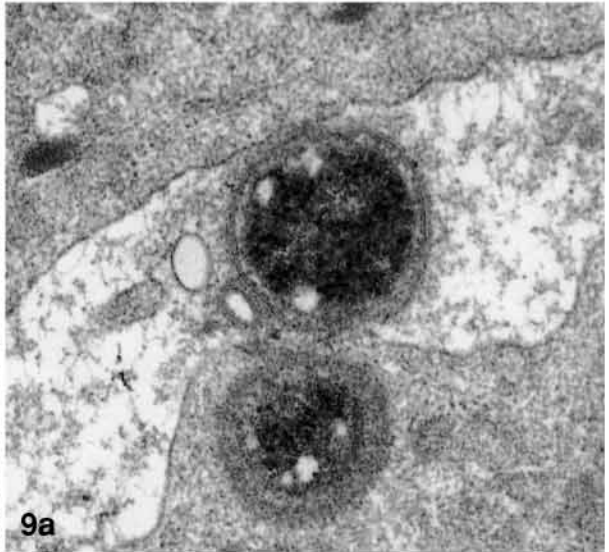
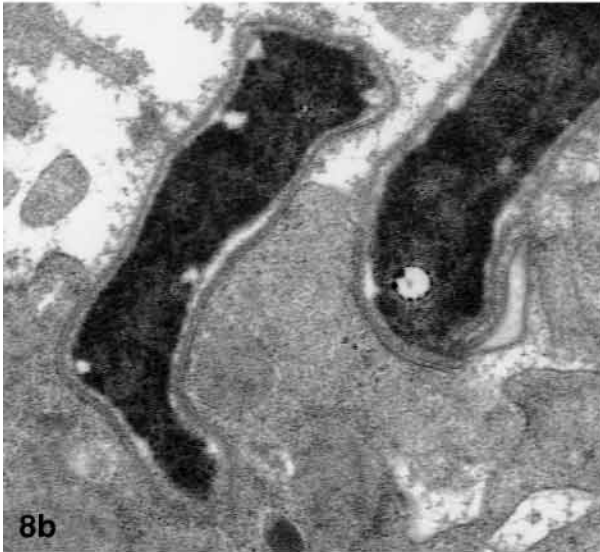
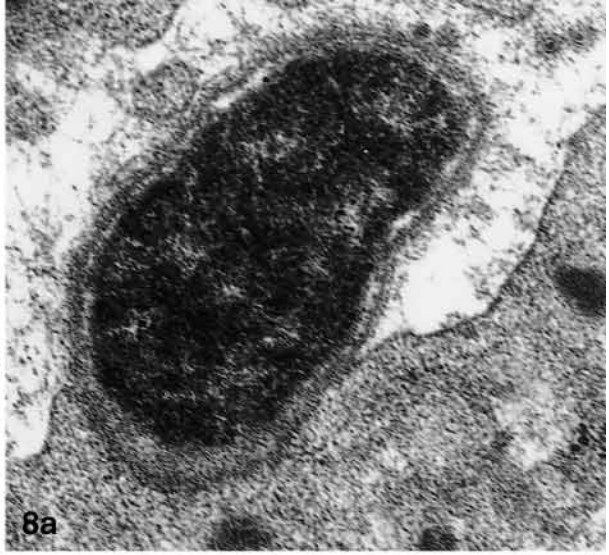
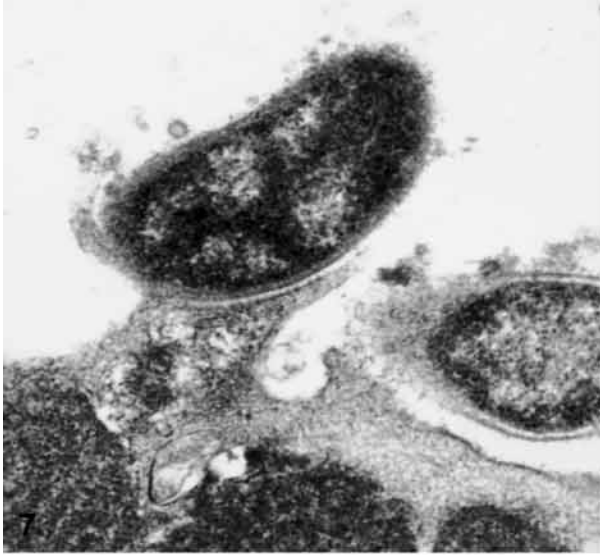
The clinical characteristics of our patients are shown in Table 1. Eight of them had active peptic ulcer disease (four in the duodenum, three in the stomach, and one had both gastric and duodenal ulcers). The other four patients showed typical histological signs of chronic active gastritis, including epithelial infiltration by polymorphonuclear leukocytes. One patient (case 3) showed an endoscopically deformed bulb, indicating previous gastroduodenal ulcer disease, and histological signs of active gastritis without obvious ulceration. In all cases, *H. pylori* was demonstrated in abundance within the surface mucous layer (Fig. 1), in accordance with previous observations. The organisms were predominantly spiral-shaped and often displayed translucent vacuole-like spaces (Fig. 1–4), probably corresponding to polyphosphate energy stores (25). Numerous bacteria were often seen in close proximity to the surface of gastric epithelial cells, as well as near or within the intercellular junctions (Figs. 2 & 3).

The intimate contact of *H. pylori* and gastric cells with practically no intervening space was termed close cell adhesion/adherence (3). An alternative form of “loose” attachment, probably through fibrillar-like strands, “anchoring” the bacteria to the gastric cells, was also observed (Fig. 5). Various forms of adhesion were demonstrated in the biopsies of 10 patients (77%). These included all peptic ulcer disease patients and one (of four) type B gastritis cases (Table 1). The most usual pattern of adhesion was the apposition of a broad area of *H. pylori* organisms to the gastric cell surface directly or via microvilli (Fig. 4). Villi appeared overall blunt, sometimes flat, resembling the “effacing” effect previously observed (1, 17). *H. pylori* were also observed within or near the intercellular junctions, occasionally in large numbers (Fig. 3). Frequently, a tight cell attachment of *H. pylori* was achieved via specialised protrusions of the gastric cell cytoplasm, so-called pedestal

*Fig. 7.* Adhesion of *H. pylori* to gastric mucous cells via cup-like formation. TEM ×39,000.

*Fig. 8.* Engulfment of *H. pylori* with partial (a) and near complete (b) fusion of the bacterial surface and the gastric cell membrane. The apical cytoplasmic region shows irregular condensation. TEM ×71,000.

*Fig. 9 (a–c).* *H. pylori* internalised by gastric epithelial cells. The organisms shown do not demonstrate any obvious degenerative changes or association with mucous granules. Some bacteria are found within the apical region (a), just beneath the gastric cell membranes. TEM ×71,000.



formations (Fig. 6). The surface of the pedestals was usually flat, but sometimes appeared curved, or cup-like (Fig. 7). Occasionally, this cup-like formation was configured as a nearly complete invagination with engulfment of the organism (Fig. 8). In some cases, the outer membrane layer of the bacterium was clearly attenuated and apparently “fused” with the gastric cell membrane.

It must be noted that several of the above-mentioned adherence forms were seen to co-exist in biopsies from the same patient. Moreover, in individual patients, *H. pylori* organisms sometimes showed several types of adherence, for example a combination of “anchorage type” and pedestal formation (Fig. 6). In the biopsies from three patients with active and from one with past peptic ulcer disease we were able to demonstrate *H. pylori* organisms within the cytoplasm of the gastric cells (Fig. 9).

The bacteria were obviously viable without any signs of degeneration, and were found at various distances from the cell membrane. No apparent association with lysosomes or mucous granules was noted for the internalised *H. pylori*, in contrast to some previous observations (22). On the other hand, in the cytoplasm of gastric cells we occasionally observed electron-dense irregular or rounded structures which could represent degenerative bacterial forms, but did not display unequivocal *H. pylori* morphology (not shown).

## DISCUSSION

The importance of *H. pylori* infection for the development of gastric inflammation, gastroduodenal peptic ulcer disease and gastric cancer, including mucosa-associated lymphoid tissue lymphoma in the human stomach, is now generally accepted (15, 26). The results from the overwhelming majority of studies (reviewed in 16, 27, 28) further converge to suggest that adherence of *H. pylori* to gastric epithelial cells is an important virulence trait, as it is for many enteric bacterial pathogens.

Subsequent pathogenic effects of the organism may be ensured by direct mechanism(s) connected with the adherence per se, amplified by the production and cell release of toxins such as vacuolating toxin and perhaps various enzymes

(29). Thus, the presence of different cell adherence forms of *H. pylori* has been correlated with cytolysis and disintegration/destruction of the gastric epithelium (3, 11, 13). Another feasible indirect mechanism of action is the recruitment and activation of inflammatory cascade(s) with potent chemokines (15).

Adherence of *H. pylori* is apparently specific to gastric epithelial cells, and was not observed with esophageal epithelial cells or gastric fibroblasts (9). Moreover, cell adherence was documented with the coccoid as well as the spiral forms of *H. pylori*, suggesting a possible importance of both forms in *H. pylori* pathogenicity (24).

The precise molecular and biochemical characteristics of *H. pylori* cell adhesion are not known and the binding interactions involve specific cell surface structures containing glycosylated proteins or lipids (27, 30), phospholipids (30) and highly sulphated molecules such as sulphatides and heparan sulphate (30, 31).

Previous studies have addressed the ultrastructural characteristics of the interaction between *H. pylori* and gastric surface cells in some detail. However, the results have often been discordant and in part difficult to interpret: different study models were applied, involving animal cells (14), cultured human gastric cells (9), gastric cancer cell lines (17, 21, 31), as well as tumour cell lines of other origin (18, 19, 22). Some investigations used live cells from patients with dyspepsia (3, 10) or gastritis (5, 7, 8, 11, 13), and only a few have included test materials from patients with gastric or duodenal ulcers (6, 20). Studying adherence of *H. pylori* using cell lines, especially of non-gastric origin, has obvious limitations. In animal models of *H. pylori* infection, neutrophilic response or ulcer in the mucosa may not be induced (14). Furthermore some of the reported discrepancies may be caused by sampling error(s), of a biological or artefactual nature, or compounded by virulence differences between strains of *H. pylori*.

*H. pylori* colonise the mucous layer close to the surface of gastric mucous cells (Fig. 1). Thomsen *et al.* (7) could demonstrate only loose attachment of the organisms and suggested that cell adherence is probably not an important virulence trait. In contrast, most other studies, using scanning or transmission electron microscopy, were able to show various forms of firm



attachment between *H. pylori* and gastric cells, often resulting in deformation, blunting or focal ablation of gastric microvilli (8, 12, 15, 17, 23). This was comparable to, but possibly biochemically different from, the "attaching-effacing" effect observed with *E. coli* strains carrying the *eae*-gene (EPEC-strains; 12, 17). The specialised attachment sites on gastric cells were described as "abutting" (3, 10), "pedestal" (8, 11, 12, 24), or "cup-like" (8). However, some investigators failed to show such pedestal formations (4). On the other hand, it was noted that more than one adhesion form could be encountered within the same biopsy material and sometimes an individual *H. pylori* organism displayed several adhesion forms simultaneously (3, 10, 12; Figs. 4 & 5). Genta et al. (23) found that on rare occasions *H. pylori* showed adhesion to areas of incomplete intestinal metaplasia with effacement of the surface villi. Ansorg & Schmid (32) recently demonstrated that contact between *H. pylori* and yeast cells is effected through "knob-like" surface attachment structures, suggesting that adhesion is a general inherent property of the bacterium.

Our results using live cells from peptic ulcer and gastritis patients confirm the presence of most previously described adhesion forms, including loose adherence ("anchorage"), with fibrillar-like strands (Fig. 5), "abutting" (Fig. 3 & 4), as well as pedestal (Fig. 6) and cup-like (Fig. 7) formation. Our finding that in all ulcer patients (including the one with healed ulcer) one or more adhesion forms were invariably present strongly indicates that firm attachment to gastric epithelium is closely associated with the pathogenicity of *H. pylori* in gastroduodenal ulcer disease. Smoot et al. (9) and Segal et al. (12) convincingly demonstrated that adhesion of *H. pylori* is followed by distinct biochemical alterations in the cytoplasm of gastric cells. These include cytoskeleton rearrangements with polymerization of actin, a-actinin and talin, and phosphorylation of host cell proteins (9, 12, 29).

Early results (6) suggested that *H. pylori* could invade the intercellular space with apparent weakening of the intercellular junctions and possible disruption of neighbouring gastric cell membranes (8). This was confirmed by many workers (4, 5, 8, 10, 15, 23) but disputed by others (7, 14). Our results showed typical and often abundant *H. pylori* in the intercellular

space (Fig. 4), but without obvious destruction of the gastric epithelium. Moreover, we observed that in some cases where *H. pylori* was engulfed by epithelial cells (Fig. 8) the outer membrane of the organism apparently merged with the cytoplasmic gastric cell membrane and both membranes appeared attenuated. This might represent the first step in the process of internalisation or endocytosis of *H. pylori* (10, 11, 27).

Our results corroborate previous observations demonstrating intracellular presence of *H. pylori* in gastric epithelial cells (4, 6, 8, 10–12, 14, 20–22). We found unambiguous internalisation of *H. pylori* in the biopsies of four patients, three with active and one with past gastroduodenal ulcer disease. Additionally, in several cases we found possible degenerate forms of the bacteria in intracellular locations, but these cases were discounted. Although our material is relatively small, the data clearly suggest the possibility that internalisation of *H. pylori* is related to the pathogenic mechanism of ulcer formation. However, we could not find internalised organisms in five out of nine (55%) ulcer patients, indicating that there is no direct correlation between this phenomenon and the development of ulcers. Furthermore, previous observations suggest that internalisation is not confined to ulcer patients. The emerging biochemical variability may be related to genetic differences between strains of *H. pylori* (26, 29, 30). Individual strains may be endowed with specific molecular characteristics facilitating adhesion to gastric cells and cell invasion. The exact biological significance of internalised *H. pylori* is not clear. The organism can obviously survive within polymorphonuclear leukocytes without being killed (33), which may be connected with the acquisition of resistance to antibiotic therapy (19). Alternatively, partial degradation and exocytosis of *H. pylori* constituents could contribute to the antigenicity of the organism. Su et al. (34) recently showed that adherence to and invasion of gastric epithelium by *H. pylori* induces tyrosine phosphorylation of proteins in focal zones. Subsequent clustering of integrins within these areas was thought to contribute to invasion and possibly the ability of *H. pylori* to establish persistent infection. Further studies are required to elucidate these alternatives and to establish the suggested link



between *H. pylori* adherence/internalisation and pathogenicity in human ulcer disease.

The paper is dedicated to our friend and colleague Associate Professor Adel Gad who died on 8 October 1998.

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